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SHEDDING OF *COXIELLA BURNETII* IN MILK OF DAIRY CATTLE AND EVIDENCE OF Q FEVER IN DOMESTIC RUMINANTS WITH EMPHASIS ON ABORTION CASES IN LATVIA

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Q-fever is widespread globally. The goal of this study was to estimate the prevalence of Q-fever infection in Latvia among dairy cattle in 2018–2020, in comparison with that in 2012–2015. The shedding of Coxiella burnetii *DNA in milk was assessed. Screening of blood samples of abortion cases for the presence of antibodies against Q-fever and testing of aborted fetuses for* C. burnetii *DNA were also carried out. Additionally, serum samples from clinically healthy cattle, sheep and goat were included. Overall, 18.34% of milk samples were positive, representing 11.02% of all tested sheds. In total, 20.62% of serum samples from the cattle that suffered abortions were positive or suspicious, representing 12.63% of all sheds. Only 3.33% of serum samples from clinically healthy cattle and 3.42% from sheep and goat were positive. The highest proportion of serologically positive and suspicious samples was observed in cattle that suffered abortion with age from three to nine years, and in the first and third gestation period. One dominant genotype of* C. burnetii *(MST61) was detected. Ruminant import from abroad was identified as a significant risk factor, as well as the dairy cattle population density.*

Keywords: Coxiellosis, dairy cows, sheep, goat, reproduction disorders, PCR, detection of immune response.

INTRODUCTION

Q fever (coxiellosis) is a zoonotic infection that has been recorded all over the world, excluding New Zealand. Its causative agent *Coxiella burnetii* can infect a broad spectrum of animals, as well as humans. *C. burnetii* has two antigenic phases — Phase I (PhI) and Phase II (PhII). PhII antibodies are produced in acute infection, while PhI — during chronic infections in humans. In cattle, a $PhI^-/PhII^+$ antibody test result indicates the acute infection, and a transition to $PhI^+/PhII^+$ status indicates the chronic infection (Böttcher *et al*., 2011). Cattle, sheep, and goats are the main reservoirs of *C. burnetii* (OIE, 2019). In cows, ewes, and goats Q fever causes late term abortions, other reproductive disorders, and metritis — and in cattle infertility can occur (De Biase *et al*., 2018). However, the infection in cattle can often be subclinical for weeks to several months (Böttcher *et al*., 2011; Guatteo *et al*., 2011). Domestic ruminants are considered to be frequent subclinical carriers that may shed bacteria in secretions and excreta (OIE, 2019). Shedding in milk can be persistent or sporadic. Persistent shedders have been characterised as persistently highly seropositive (Guatteo *et al*., 2007). In Germany, the highest antibody prevalence (> 30%) was observed in cows aged from seven to eight years,

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but in general it was above 25% in age groups above four years (Böttcher *et al*., 2011). Significant increase in seropositivity in cows was observed starting from the age above two years in Northern Ireland (McCaughey *et al*., 2010). In some investigations, the disease prevalence in cattle herds was linked to abortions (Cetinkaya *et al*., 2000; Cabassi *et al*., 2006). For example, in Italy 44.9% of cattle that suffered abortions were seropositive in comparison to a control group in which only 22% of animals were seropositive (Cabassi *et al*., 2006). In Latvia, the prevalence of *C. burnetii* antibody — positive sheds in 2012–2015 was 13.4% among the investigated abortion cases (Boroduske *et al*., 2017).

While cows often can be asymptomatic and seronegative, they may still shed *C. burnetii* DNA in milk (Rodolakis *et al*., 2007). Analysis of *C. burnetii* DNA in bulk tank milk (BTM) samples is useful for estimation of the disease prevalence at a regional or country level. In a study carried out in 2007 in the Netherlands it was found out that 56.6% of all tested herds had bacterial *C. burnetii* DNA in BTM samples (Muskens *et al*., 2011). In the Bizkaia province of northern Spain, 51.7% of herds were positive for *C. burnetii* by BTM PCR (Astobiza *et al*., 2012). Shedding of the pathogen via milk has been recorded to be as high as 31.54% of tested dairy cattle herds in Poland (Szymańska-Czerwińska *et al*., 2019) or even 69.7% in south-west England (Valergakis *et al*., 2012). In a previous study in Latvia in 2015, 10.7% of dairy cow sheds tested positive for the presence of *C. burnetii* DNA in BTM samples (Boroduske *et al*., 2017).

The presence of *C. burnetii* in BTM in Netherlands showed correlation with various risk factors, including the purchase of cattle from at least two different locations, the presence of tick infestation, the use of an automatic milking systems and the presence of goats or sheep on the farm (van Engelen *et al*., 2014). Large herd size (100 and 200) and dairy breeds were identified as significant risk factors in Northern Ireland and the Republic of Ireland (McCaughey *et al*., 2010; Ryan *et al*., 2011). Environmental factors like high winds and low precipitation were identified as risk factors in Sweden (Nusinovici *et al*., 2015). Sheep and goats were shown to be a major source of Q-fever outbreaks in the human population (EFSA Panel on Animal Health and Welfare, 2010) and a major risk factor for Q-fever in cattle (Nusinovici *et al.*, 2015; Plummer *et al*., 2018). Herd size, the location of the cattle within a risk area, and purchasing cattle from other countries were identified as significant risk factors in Latvia (Boroduske *et al*., 2017).

For epidemiological reasons, genetic characterisation of *C. burnetii* is necessary (Sulyok *et al*., 2014). Using the multispacer sequence typing (MST) of genetically variable intergenic regions, initially 30 different allelic combinations were obtained (Glazunova *et al*., 2005). Currently there are more than 70 MST groups distinguished (https://ifr48.timone.univ-mrs.fr/mst/coxiella_burnetii/strains.html). A study in Portugal on *C. burnetii* genotypes prevalent in different municipalities of the country showed that different genotypes were present in acute and chronic disease cases. Acute disease in humans in the Évora, Faro, Lisbon, and Setśbal municipalities was caused by the MST4 genotype, while in goats in the Castelo Branco municipality it was caused by MST13. In contrast, chronic human disease in the Castelo Branco, and Lisbon municipalities was caused by MST8 (Santos *et al*., 2012). Major outbreaks in the human population in the Netherlands were caused by MST33, as in infected sheep and goats (Tilburg *et al*., 2012). A study in Poland revealed a new genotype in both pathological samples from dairy cows and in marketed dairy products. Detailed analysis of the new MST61 genotype showed the highest similarity to MST20 (Szymańska-Czerwińska et al., 2019). MST20 is found in cattle in many countries, and currently is the predominant sequence type recorded in bovine milk samples in the United States (Person *et al*., 2014; Bauer *et al*., 2015).

The goal of this study was to estimate the prevalence of Q-fever infection in Latvia by assessing the shedding of bacterial DNA in BTM and pooled milk samples, the presence of *C. burnetii* DNA in aborted foetuses, and the presence of *C. burnetii* antibodies in serum samples from cattle that suffered abortion over the years 2018–2020, in comparison to previous results from a study conducted in 2012–2015. Additionally, serum samples from clinically healthy cattle, sheep and goats were included in the study. During this time period, no special nationwide Q-fever prevention measures or control programmes were implemented in the country level. However, all animal owners were informed by the national veterinary authorities when abortion due to *C. burnetii* infection was found and the possible measures were discussed. During the years 2015–2020, the average number of cattle remained without significant changes — around 400 thousand. However, the total number of dairy cow sheds decreased from 16 thousand in January 2018 to 11.8 thousand in December 2020 (Central Statistical Bureau of Latvia, 2019; Agricultural Data Centre, 2020). Although no registered human Q fever cases were reported during the years 2016–2019, one case was identified in the first quarter of 2020 (Centre for Disease Prevention and Control of Latvia, 2020).

Several potential risk factors were assessed in order to evaluate their impact on the prevalence of the disease in particular regions of the country — the density of ruminants, dairy cows and small ruminants, and the number of imported ruminants. The dominant genotypes of *C. burnetii* were detected by multispacer sequence typing (MST).

MATERIAL AND METHODS

Samples. The study material included 1505 BTM and pooled milk samples from 1062 dairy cattle sheds submitted to the Institute of Food Safety, Animal Health, and Environment "BIOR" under the National Programme for the Control of Bovine Leucosis Virus and *Brucella* sp. surveillance programme during the period from December 2018 to June 2019 and from December 2019 to December 2020 (Table

Table 1. The number and type of samples included in the study (2018–2020)

Sample type	Serum from aborted cattle	Pathological material	Bulk tank milk and pooled milk	Serum from clinically healthy cattle	Serum from sheep and goats
Number of samples (n)	1557	744	1505	300	117
Number of represented sheds	573	308	1062	32	31
Number of local regions	79	68	90	29	23

1). Altogether milk samples from 35 408 cattle were analysed. The milk samples were pooled with up to 50 animals per pool. The analysed sheds represented 90/110 (81.82%) of all local regions in Latvia. Milk samples with added 2-bromo-2-nitropropane-1,3-diol were transported to the laboratory in a cold box at 4 to 6 °C temperature and processed or stored for one to seven days in a refrigerator at the same temperature before laboratory testing.

In total, 1557 dairy cow blood samples from cattle that suffered abortion, representing 573 dairy cattle sheds and 744 samples of pathological material from aborted foetuses, including spleen, liver of aborted foetus, and placenta, representing 308 sheds were sampled during the three-year period of January 2018 – December 2020 (Table 1). The samples were collected from cases of cattle abortions under the National Dairy Cattle Abortion Surveillance Programme. Information on the gestation period was indicated for the time of abortion or stillbirth in 663 abortion cases.

Additionally, 300 serum samples from clinically healthy dairy cattle (control group) and 117 serum samples from sheep and goats were included in the study. Both groups were evenly distributed around the whole country. According to the age distribution of aborted dairy cattle, the control group of dairy cattle, and sheep and goats, 60–70% of the animals had age of 2–5 years. All dairy cattle from both groups belonged to the female gender.

In total, 4106 samples from 1610 dairy cattle sheds were tested during this study, representing 13.64% of all dairy cattle sheds in the country.

DNA extraction and amplification. Suspensions from pathological material, including spleen and liver of the aborted foetuses, as well as placenta, were prepared for DNA extraction. A total of 200 µl of tissue suspension in transport medium or PBS buffer was used for DNA isolation. If several internal organs (liver, lungs, and spleen) were available from one aborted foetus, the suspensions were pooled to a total volume of 200 µl before DNA extraction.

Swabs from the placental samples were taken by rubbing three cotton balls against the placental cotyledons at three different sites. If necessary, incisions were made with a sterile scalpel to access the swabbing site. The swabs were placed into one tube of transport medium. The tube was vortexed for 30 seconds, and a 200 µl aliquot of smear suspension was taken for DNA isolation.

The manual extraction of DNA from the suspensions and milk samples for real-time PCR was performed with a QIAamp DNA Mini kit (Qiagen, Germany) using 100 µl elution volume. The ADIAVET Cox Real time qPCR kit (Bio-X Diagnostics, France) according to the supplied protocol was used to amplify the IS1111 DNA sequence and GAPDH, as an internal control of extraction and amplification steps, with quantification using a Rotor-Gene Q real-time PCR cycler (Qiagen, Germany). According to the manufacturer's data, the PCR detection limit is 1.5 *C. burnetii* / 5 µl of DNA in PCR, and the PCR quantification limit is 2 *C. burnetii*/5 µl of DNA in PCR. Using the ADIAVET Cox Real time qPCR kit, the milk of one *C. burnetii* shedding cow can be detected in a pooled milk sample from up to 250 cows (Barberio, 2015).

Serological analysis. All blood samples were screened for the presence of antibodies against *C. burnetii* by ELISA, using the ID Screen Q Fever Indirect Multi-species kit (IDvet, France). The results for serum samples were interpreted according to the manual provided by the manufacturer. A serum sample was determined to be positive if S/P% > 50%, a sample was suspected to be positive if 40% > S/P% ≤ 50%, and negative if S/P% ≤ 40%. According to the information provided by the manufacturer the sensitivity and specificity of the test is 100%. Part of the serum samples from cows that suffered abortion (n = 294, including 202 positive and suspicious samples tested with IDvet ELISA kit and 92 negative samples) were analysed with VetLine Coxiella Phase I and Phase II kits (NovaTec, Germany) that have been validated by the manufacturer for research use only. According to the information provided by the manufacturer, the sensitivity of both tests is 96.20% and the specificity of both tests is 94.47%.

Detection of *C. burnetii* genotype by MST. Strongly positive samples (n = 26) with at least 5×10^4 genome equivalents / ml (Ct values < 27) were selected for genotyping and 10 intergenic spacers were used — Cox2, Cox5, Cox18, Cox20, Cox22, Cox37, Cox51, Cox56, Cox57, and Cox61, as described previously (Glazunova *et al*., 2005). The forward primer of Cox57 was modified as described previously (Di Domenico *et al*., 2014). The obtained sequences were processed using Bioedit software (Tippmann, 2004) and were identified by comparison with the sequences deposited in the Méditerranée Infection Foundation database (https://ifr48.timone.univ-mrs.fr/mst/coxiella_burnetii/strains.html) and published in other studies (Mioni *et al*., 2020; Sulyok *et al*., 2014; Gyuranecz *et al*., 2014). For clustering analysis and exploration of genotype diversity, the MSTree V2 method was used with GrapeTree (Zhou *et al*., 2018).

Statistical analysis. Cartographic representation of the obtained results was done with QGIS free access software Version 3.18.1 (https://www.qgis.org/en/site/). Additional

information was obtained from the public database of the Agricultural Data Centre, including the number of ruminants, dairy cows, sheep, and goats in the local region, and the number of imported ruminants in the local region (Agricultural Data Centre, 2020).

Statistical analyses were performed using the chi-squared test (Social Science Statistics, 2020), and the 95% confidence interval for disease prevalence was calculated by the WilsonCL algorithm (Brown *et al*., 2001) using the open access program Epitools (https://epitools.ausvet.com.au/trueprevalence). Stepwise multiple regression analysis was conducted using the R base package (R Development Core Team, 2009). The following risk factors were included in the analysis: the population density of ruminants, dairy cows and small ruminants, and the number of imported ruminants in the local region.

A comparison of disease status data between time periods of years 2012–2015 and 2018–2019 was done by classification of sheds into three categories: A – all tested indicators were positive, B – at least one of the tested indicators was positive, and C – all tested indicators were negative (according to Piñero *et al*., 2014).

RESULTS

In total, 20.62% of serum samples from cows that suffered abortions were positive (95% confidence interval (CI): 18.68–22.70). Only 3.33% of serum samples from clinically healthy cattle were positive (95% CI: 1.82–6.03), representing 15.63% of all sheds from this sample group (Table 2). The differences in seropositivity between cows that suffered abortions and this control group were statistically significant: χ^2 with Yates correction was equal to 50.12, $p < 0.00001$. Part of the serum samples from cows that suffered abortions were analysed with VetLine Coxiella Phase I and Phase II kits. Of the samples that were positive or suspicious according to the IDvet ELISA kit, 97 samples were negative or suspicious with NovaTec phase kits. From all tested samples, 13 samples were positive for $PhI^+/PhII^+$ antibodies (4.42%, (95% CI: 2.60–7.42), 62 samples (21.09%, 95% CI: 16.81–26.11) for $PhI^+/PhII^-$, and 12 samples (4.08%, 95% CI: 2.35–7.00) for $PhI^-/PhII^+$. From the ten clinically healthy cattle serum samples that were IDvet ELISA kit positive, four were $PhI^+/PhII^-$ and two samples were $PhI^+/PhII^+$. Among the four IDvet ELISA kit positive sheep and goat blood serum samples, only one sample was positive for both PhI and PhII antibodies, and the others were negative with these tests.

Only 28 samples of the pathological material from aborted foetuses, corresponding to 3.76% (CI: 2.62–5.39), were positive for Q-fever DNA by PCR test. Of these cases, fifteen samples were from one shed, from which 142 blood serum samples from cows that suffered abortions were submitted. For 21 of the 28 PCR positive cases, either abortion during gestation period III or stillbirth had occurred. In nine cases of 28, the blood serum of the cows that suffered abortions did not show antibodies against Q-fever, and in three additional cases the ELISA IDvet test results were suspicious. The copy numbers of serologically positive samples ranged from 8.8×10^1 to 6.2×10^4 *C. burnetii* genome equivalents per g. For serologically suspicious samples, the copy numbers ranged from 3.8×10^2 to 1.8×10^3 *C. burnetii* genome equivalents per g (GE/g), and for serologically negative samples — from 8.1×10^1 to 3.5×10^3 *C. burnetii* GE/g.

In total, 18.34% of BTM and pooled milk samples were positive (95% CI: 16.47–20.37). The positive milk samples represented 9877 of dairy cattle. The *C. burnetii* DNA copy numbers of positive samples ranged from 0.4×10^1 to 1.1×10^6 *C. burnetii* genome GE/ml. Altogether positive cattle sheds were detected in 47 local regions.

Sheep and goat blood serum samples were serologically positive in 3.42% of cases (CI: 1.34–8.46) and represented one sheep shed and one goat shed (Table 2).

Spatial distribution data for all tested and positive dairy cattle sheds are given in Figures 1 and 2. The increased risk area for the seroprevalence in blood serum of dairy cows after abortions in the recent studied period was similar to that in the first study from years 2012–2015, but shedding via milk had increased in other parts of the country outside of the previous high-risk area identified as the northern part of Latvia.

Stepwise multiple regression analysis showed that none of the risk factors included in the analysis (the populations density of ruminants, dairy cows, and small ruminants, the number of imported ruminants from abroad in the local region) had a statistically significant effect on the number of positive sheds in the local region (detected by PCR of milk samples or positive serum samples according to the IDvet test). The dairy cattle population density in the local region and the number of imported ruminants from abroad in the

Table 2. The number and proportion of positive samples, sheds and local regions (2018–2020)

Sample type	Number of samples (n)	Number of positive samples (%, 95% CI)	Number of positive sheds (%)	Number of positive local regions (%)
Bulk tank milk and pooled milk samples	1505	276 (18.34, 16.47 – 20.37)	117 (11.02)	47 (52.22)
Pathological material from aborted foetuses	744	28 (3.76, 2.62 – 5.39)	5 (1.62)	4 (5.88)
Serum samples from cows that suffered abortions	1557	321 (20.62, 18.68 – 22.70)*	73 (12.74) *	30 (37.97) *
Serum samples from clinically healthy cattle	300	10 (3.33, 1.82 – 6.03)*	5 (15.63) *	5 (17.24) *
Sheep and goat blood serum samples	117	4 (3.42, 1.34 – 8.46)	2 (6.45)	2 (8.70)

* Together with serologically suspicious samples.

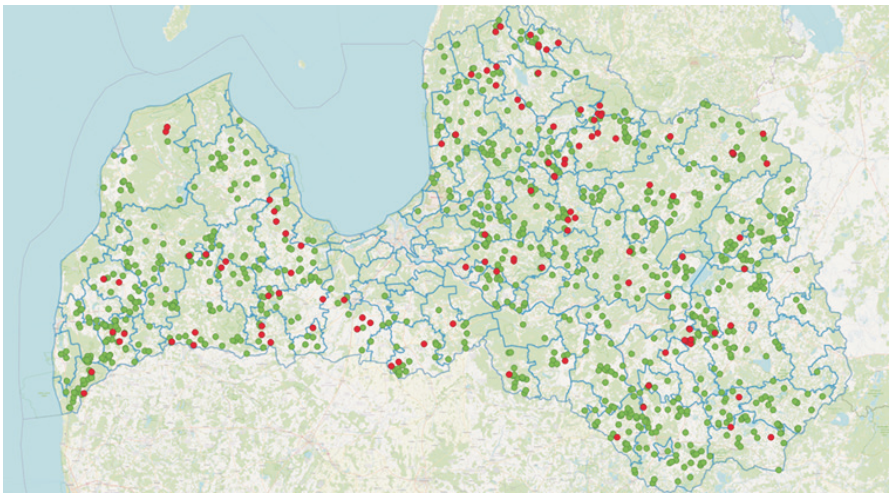


Fig. 1. The spatial distribution of dairy cow sheds analysed during the years 2018–2020 for *Coxiella burnetii* DNA in milk samples. The green dots on the map indicate the locations of sheds where all samples analysed were negative, while the red dots indicate those holdings where at least one positive sample was found.

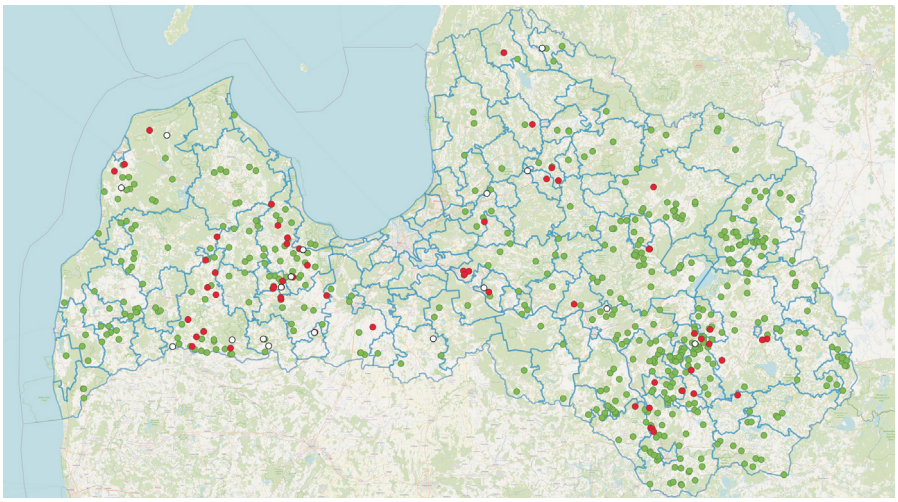


Fig. 2. The locations of dairy cow sheds analysed during the years 2018–2020 for *Coxiella burnetii* antibodies in blood serum of cows that suffered abortions. The green dots on the map indicate the locations of sheds where all samples analysed were negative, the red dots indicate sheds where at least one positive sample has been found, and white dots indicate sheds where at least one suspicious sample was found.

local region were associated with the total number of positive milk samples in the local region. The population density of sheep and goats in the local region had no significant effect on the total number of positive milk samples in the local region, but the total ruminant density in the region had negative effect on the total number of positive milk samples in the local region (Table 3).

The changes in seroprevalence according to the IDvet test among cows that suffered abortions showed decrease in absolute numbers of positive and suspicious animals over the study years but did not show a decrease in the percentage of serologically positive cattle (Table 4).

The analysis of the results according to the IDvet test of the serum samples from cows that suffered abortions by age

Table 3. Results of stepwise multiple regression analysis of risk factors and number of positive milk samples

Variable name	Coefficient ± SE	*t* value	*p* value
Intercept	3.07 ± 1.49	2.06	0.045
Ruminant population density in the region, animals/km^2	–1.87 ± 0.50	–3.74	0.0005
Dairy cattle population density in the region, animals/km^2	5.84 ± 1.18	4.93	0.00001
Density of sheep and goat population in the region, animals/km^2		Not significant	
The number of imported ruminants from abroad in the local region	0.009 ± 0.003	3.38	0.001
p value of the final model is 3.3×10^{-9}, multiple R^2 is 0.62			

Table 4. The changes in seroprevalence among cows that suffered abortions according to the IDvet test

Test result	2018		2019		2020	
	I-VI	VII-XII	I-VI	VII-XII	I-VI	VII-XII
Positive (%)	62 (16.58)	52 (20.16)	70 (21.34)	50 (22.42)	30 (13.45)	31 (20.53)
Suspicious (%)	5 (1.34)	5 (1.94)	6 (1.83)	3 (1.35)	5 (2.24)	2 (1.32)
Negative (%)	307 (82.09)	201 (77.91)	252 (76.83)	170 (76.23)	188 (84.30)	118 (78.15)
Total	374	258	328	223	223	151

Table 5. Results of serum samples from aborted cattle by age according to the IDvet test

Age, years	0-1	2	3	4	5	6	7	8	9	10	> 10
Total number of samples	52	244	362	293	189	170	120	49	39	17	21
Positive/ suspicious	5/3	30/4	83/3	59/7	42/1	33/5	23/3	8/0	8/0	3/0	1/0
Proportion, %	15.4	13.9	23.7	22.5	22.7	22.3	21.7	16.3	20.5	17.6	4.8

Table 6. The presence of *C. burnetii* antibodies depending on cattle gestation period (n = 660) according to the IDvet test

Gestation period	Number of investigated samples	Number of seropositive and suspicious samples	Proportion of seropositive and suspicious samples, %
I (1–3 months)	65	24	36.92
II (4–6 months)	278	58	20.86
III (7–9 months)	317	112	35.33

categories indicated that the highest proportion of positive and suspicious samples (> 20%) was observed in the age group from three to seven years and at the age of nine years (Table 5). The presence of *C. burnetii* antibodies depending on gestation period of cows that suffered abortions is summarised in Table 6, showing the highest percentage of positive and suspicious samples in the first and third gestation periods.

A comparison of positive milk or serum test incidence data between the time periods of 2012–2015 and 2018–2019 by classification of sheds into three categories A, B, and C according to the test results in 2021–2015 indicated that a small portion of the category A sheds (with all tested indicators positive) from the first time period (1.22%) remained positive during the second time period, but 1.84% showed no positive samples in the second period. Similarly, a small portion of infected farms (2.35%) was detected in the second time period that were negative in the first time period (Table 7).

Genotypic characterisation with the MST method indicated that there was only one MST type present in the country — MST61. It was possible to obtain a complete allelic profile only for two of the 26 samples. For the other samples, the genotype was determined on the basis of incomplete allelic profiles. All samples showed identical profiles of sequenced intergenic spacers, with the exception of one sample that had Cox2.7 instead of Cox2.3 (Table 8).

The sequences obtained during the present study were compared with other publicly available sequences from other countries (Figs. 3 and 4). The MST61 genotype found in this study was identical to that found in Poland and Iran. Very similar genotypes with a difference of only 1 allele have been found outside of Europe.

DISCUSSION

This study estimated the prevalence of Q-fever in the territory of Latvia by assessing the shedding of bacterial DNA in BTM and pooled milk samples, the presence of *C. burnetii* DNA in aborted foetuses, and the presence of *C. burnetii* antibodies in serum samples from cows that suffered abortion over the years 2018–2020. The results were compared with those from a previous study conducted in 2012–2015. Additionally, serum samples from clinically healthy cattle, sheep, and goats were included in the study. During this time period, no special nationwide Q-fever prevention measures or control programmes were implemented at the country level, and solutions were applied only on a case by case basis. In accordance with the Regulations of

Table 7. The changes in *C. burnetii* status between the years 2012–2015 and 2018–2020

Category in 2012 – 2015	Number of sheds (%)	Number of positive samples (detection method)				Category in 2018 – 2019	Number of sheds (%)
		Serum (ELISA)	Milk (ELISA)	Milk (PCR)	Pathological material (PCR)		
A	99 (10.10)	82	20	20	4	A	12 (1.22)
						B	14 (1.43)
						C	18 (1.84)
						Unknown	55 (5.61)
B	25 (2.55)	9	13	7	0	A	4 (0.41)
						B	4 (0.41)
						C	3 (0.31)
						Unknown	14 (1.43)
C	856 (87.35)	0	0	0	0	A	23 (2.35)
						B	13 (1.33)
						C	182 (18.57)
						Unknown	638 (65.10)
Total	980	91	33	27	4		980 (100.00)

A, all tested indicators were positive; B, at least one of the tested indicators was positive; C, all tested indicators were negative.

Table 8. The identified alleles of intergenic spacers and the corresponding MST type of strongly positive samples

Sample type	Local region	The identified alleles of intergenic spacers Cox										MST type
		2	5	18	20	22	37	51	56	57	61	
Fetus	Auces	3	2	6	1	5	10	4	10	6	5	MST61 (n = 2)
Fetus	Auces	3	2	6	1	5	10	4	-	6	5	MST61*
Fetus	Auces	3	2	6	1	5	10	4	-	-	5	MST61*
Fetus	Auces	-	2	6	-	5	10	4	-	-	5	MST61*
Milk	Auces	3	2	6	1	5	10	4	-	-	5	MST61*
Milk	Brocēnu	3	2	6	1	5	10	4	-	-	5	MST61 (n = 2)*
Milk	Brocēnu	3	2	6	-	5	10	4	-	-	5	MST61 (n = 2)*
Milk	Brocēnu	3	2	6	-	5	10	-	-	-	5	MST61*
Milk	Brocēnu	3	2	6	-	5	10	4	-	-	-	MST61*
Milk	Kocēnu	3	2	6	1	5	10	4	-	-	5	MST61*
Milk	Priekuļu	7	2	6	-	5	10	4	-	-	5	-
Milk	Priekuļu	3	2	6	1	5	10	4	-	-	5	MST61*
Milk	Riebiņu	3	2	6	1	5	10	4	-	-	5	MST61*
Milk	Tērvetes	3	2	6	1	5	10	4	-	-	5	MST61 (n = 2)*
Milk	Tērvetes	3	-	6	1	5	10	4	-	-	-	MST61*
Milk	Tērvetes	3	2	6	1	5	-	4	-	-	5	-
Milk	Tērvetes	3	-	-	1	-	10	4	-	-	5	MST61*
Milk	Tērvetes	3	-	-	1	5	10	4	-	-	-	MST61*
Milk	Tērvetes	3	-	6	1	5	10	4	-	-	5	MST61*
Milk	Tukuma	3	2	6	1	5	10	4	-	-	5	MST61*
Milk	Valkas	3	2	6	1	5	10	4	-	-	5	MST61*
Milk	Ventspils	3	2	6	1	5	10	4	-	6	5	MST61*

* Genotype determined on the basis of incomplete allelic profile.

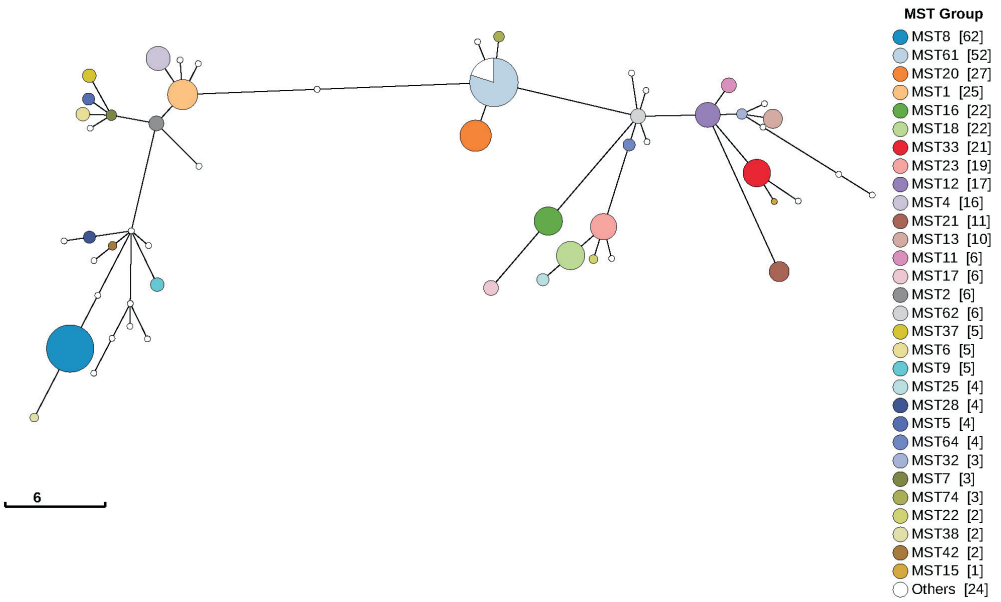


Fig. 3. The similarity of all publicly documented *Coxiella burnetii* MST genotypes. Sequences for the comparison were obtained from the Méditerranée Infection Foundation database, the present study for MST61, and several previous studies.

the Cabinet of Ministers No. 127, "Regulations on notifiable, registrable and state-supervised infectious animal diseases and the procedure for providing information thereof to the Food and Veterinary Service", Q fever is included in the list of Notifiable Animal Infectious Diseases and Infectious Animal Diseases under State Supervision. All animal owners and responsible veterinarians were informed by the national veterinary authorities when an abortion due to *C. burnetii* infection was found and possible measures were discussed and implemented.

Since the Q-fever is a zoonotic disease, from the One Health perspective, it is important to monitor the occurrence of the disease among ruminants, as well as to raise awareness among farmers and veterinarians, as under particular circumstances the presence of the causative agent in the dairy production chain may result in large human outbreaks (Eldin *et al.*, 2017; Mori and Roest, 2018; Koehler *et al.*, 2019; Rahaman *et al.*, 2019). In many countries, the sources for outbreaks in humans and cattle have been small ruminants — sheep and goats (EFSA Panel on Animal Health

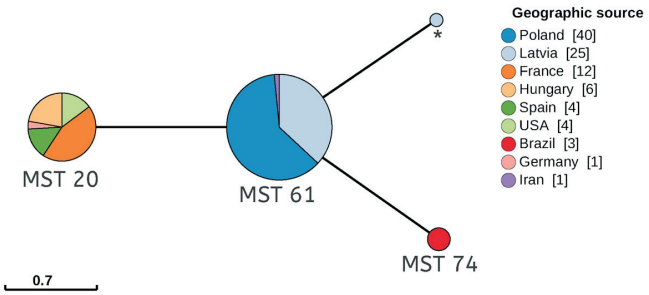


Fig. 4. A comparison of Latvian isolates with the closely related isolates from other countries, where * denotes the one isolate that had Cox2.7. Sequences for comparison were obtained from the Méditerranée Infection Foundation database, present study for MST61, and several previous studies.

and Welfare, 2010; Dijkstra *et al*., 2012; Nusinovici *et al*., 2015; Álvarez-Alonso *et al*., 2018; Clark and Magalhães, 2018; Plummer *et al*., 2018). According to the data obtained within the present study, Q-fever among sheep and goat sheds was detected only in two local regions of Latvia (Limbažu and Rēzeknes), unlike the common occurrence in dairy cattle sheds, as serologically and PCR-positive sheds were identified in 58 local regions. In the previous study, the sheep and goat population density in a local region was not a significant risk factor for positive test results from cattle sheds (Boroduske *et al*., 2017). In the present study, stepwise multiple regression analysis showed that dairy cattle density and number of imported ruminants from abroad in the local region was associated with total number of PCR positive milk samples in the local region. Cattle population density has been detected as a significant risk factor in Sweden for the occurrence of *C. burnetii* in dairy cattle herds, along with other risk factors — high wind speed, open landscape, and high temperatures (Nusinovici *et al*., 2015). A significant impact of imported animals from abroad was detected in the previous investigation as well (Boroduske *et al*., 2017). Animal trade has been identified as a significant general risk factor for Q-fever distribution in general in several studies (Pandit *et al*., 2018). It seems likely that presently in Latvia, sheep and goats do not play a significant role in the disease distribution.

In the present study, bacterial shedding via milk was common. In total, 18.34% of BTM and pooled milk samples were positive, representing 9877 animals — 7.09% of all dairy cattle in Latvia. The proportion of farms in Latvia where positive milk was found had remained at the previous level (11.02% of positive sheds vs. 10.7%) (Boroduske *et al*., 2017) and was lower than in the geographically closest countries — positive BTM test results were obtained with the same real-time PCR kit in 31.54% of sheds in Poland (Szymańska-Czerwińska *et al*., 2019), and the overall rate of positive BTM samples in Central and Eastern European countries was 40.63–44.94% (Dobos *et al*., 2020). The *C. burnetii* DNA copy numbers in positive milk samples ranged from 0.4×10^{1} to 1.1×10^{6} *C. burnetii* GE/ml. The highest values were higher than in the case of positive pathological material samples, which contained up to 6.2×10^{4} *C. burnetii* GE/g. In other investigations, dairy products have been evaluated as strongly positive when they had 3.0 log10 DNA copies/ml (Eldin *et al*., 2013). The mean bacterial load in Spain has been detected as 1.7, 2.6, and 3.3 *C. burnetii*/ml (log10 basis) in negative, slightly positive, and strongly positive BTM samples assessed by ELISA, respectively (Astobiza *et al*., 2012). In the present investigation, 57.61% of all positive milk samples had 3.0 log10 DNA copies/ml, and therefore should be recognised as strongly positive. Cattle can be asymptomatic and shed *C. burnetii* in milk more often than goats and sheep, and for longer periods (Rodolakis *et al*., 2007); therefore, BTM samples provide suitable material for detecting cattle sheds with active *C. burnetii* milk shedders (Kalaitzakis *et al*., 2021). The shedding pattern in cattle can be persistent, sporadic or intermittent (Guatteo *et al*., 2007), but other routes of shedding have to be assessed as well (vaginal mucus, faeces) to estimate the disease prevalence more accurately (Guatteo *et al*., 2006). Nevertheless, milk has been characterised as the most common and persistent shedding route (Guatteo *et al*., 2012) and shedding via milk can be associated with chronic subclinical mastitis (Barlow *et al*., 2008).

Changes over time in seroprevalence among cows that suffered abortion showed a decrease in the absolute numbers of positive and suspicious animals between the study years (114 in 2018 vs. 61 in 2020) but did not show a decrease in the percentage of serologically positive cattle, which varied from 13.45% to 22.42% (Table 4). The percentage of serologically positive sheds showed a slight decrease (12.63% *vs*. 13.4%) in comparison to the previous study period in 2012–2015 (Boroduske *et al*., 2017). In comparison, the seroprevalence of infected dairy cattle herds in the neighbouring country Lithuania was 52.15% (Rapaliutė, 2019), while 44 of 117 sheds (37.61%) in Estonia tested positive for *C. burnetii* antibodies in BTM samples (Neare *et al*., 2020). The study in Lithuania did not particularly focus on cows that suffered from abortion, but included also other types of cattle groups — for purchase or sale of cattle and suspicion of the disease (Rapaliutė, 2019). Nevertheless, that study can be considered comparable by sampling and test methods. Also, the data from the study in Estonia (Neare *et al*., 2020) are comparable with the previous study in Latvia, where BTM samples tested for *C. burnetii* antibodies showed 13.2% of positive dairy cow sheds (Boroduske *et al*., 2017).

The analysis of serum samples from cows that suffered abortion by age categories indicated that the highest percentage of positive and suspicious samples (> 20%) occurred at the age from three to seven years and at the age of nine years (Table 5). The low percentage of seropositive animals at the age of two years and younger was similar to results of studies in Northern Ireland (McCaughey *et al*., 2010) and Germany (Böttcher *et al*., 2011). In the case of Northern Ireland, it was explained by the fact that the first parturition occurs between the age of two to three years, while in Latvia the first parturition can be at younger age. In some investigations, age has been characterised as the only

significant risk factor associated with a positive serological test for *C. burnetii* (Alvarez *et al*., 2012).

When comparing the presence of *C. burnetii* antibodies depending on gestation period in cows that suffered abortion, the highest percentage of positive and suspicious samples was found in the first and third gestation periods — 35.44% and 36.93%, respectively, vs. 20.86% in the second gestation period. In total, the pathological material from aborted foetuses (spleen, liver) or placenta was PCR positive only in 28 abortion cases (3.76%), pointing to other causes of the abortion besides Q-fever, as many other causative agents of cattle abortions have been previously registered in Latvia. For example, earlier studies in Latvia have found that in more than half of abortion cases with infectious etiology, bacteria of the *Listeria* genus (*L. monocytogenes*, *L. innocua*, *L. seeligeri*) have been detected (Piginka-Vjaceslavova *et al*., 2020). In 21 of these cases, it was either in gestation period III or a stillborn calf. Higher seropositivity to *C. burnetii* in gestation period III for cows that suffered abortion has been observed in other investigations as well (Cabassi *et al*., 2006). Low frequency of potentially Q-fever related abortions in cattle in general (2.7%) have been observed in France, although the within-herd disease seroprevalence was 22.2% and between-herd seroprevalence was 36.0% (Gache *et al*., 2017). In cattle there is no experimental evidence that presence of *C. burnetii* in abortion material proves the bacteria as the cause of the abortion (Agerholm, 2013). In recent studies in Canada and Germany, the connection between the seropositivity and reproductive outcome was not statistically proved, probably due to small sample size (Freick *et al*., 2017; Turcotte *et al*., 2021).

Among the 294 serum samples tested with NovaTec phase kits, 13 (4.42%) were positive for both Phase I and II antibodies against *C. burnetii*. According to the results of other studies, $PhI^{+}/PhII^{+}$ positive antibodies indicate chronic infection with Q-fever (Böttcher *et al*., 2011) and are characteristic of chronic *C. burnetii* shedders in milk (Lucchese *et al*., 2015). In total, 12 samples (4.08%) were $PhI^{-}/PhII^{+}$, corresponding to an acute infection (Böttcher *et al*., 2011), and 62 samples (21.09%) were $PhI^{+}/PhII^{-}$. This pattern was seldom detected in a study from Germany, and a conclusion was made that it developed from the $PhI^{+}/PhII^{+}$ pattern, with the majority of these animals subsequently reverting to negative, apparently by building up an efficient cellular immunity (Böttcher *et al*., 2011).

Comparison of shed category data between time periods 2012–2015 and 2018–2019 by classification of sheds into three categories A, B, and C (A – all tested indicators were positive, B – at least one of the tested indicators was positive, C – all tested indicators were negative) indicated that 12 of the 99 category A sheds from the first period remained positive, indicating long presence of *C. burnetii* within a shed, in line with in other studies (Piñero *et al*., 2014).

Spatial distribution data for all tested and positive dairy cattle sheds (Figs. 1 and 2) indicated that the increased risk area for the seroprevalence in blood serum of dairy cows after abortions has remained similar to the first study of 2012–2015, but shedding via milk has increased also in the other parts of the country, besides the previous high-risk area in the Northern part of Latvia (Boroduske *et al*., 2017).

Genotypic characterisation with the MST method indicated that there is only one MST type present in the country — MST61. All samples showed an identical profile of sequenced intergenic spacers, with the exception of one sample that had Cox2.7 instead of Cox2.3. It was possible to obtain a complete allelic profile for only two of the 26 samples. For the other samples, the genotype was determined based on incomplete allelic profiles. This can be considered as a limitation of our study, but a reduced number of intergenic regions has also been used for the comparison of sequenced samples also in some other Q-fever studies, such as in Algeria (Rahal *et al*., 2018), Brazil and Argentina (Mioni *et al*., 2020), Senegal (Mediannikov *et al*., 2010) and Poland (Szymańska-Czerwińska *et al*., 2019).

The MST61 genotype found in our study was identical to that found in Poland and Iran. Information from other studies revealed that the MST61 genotype was recently discovered in Poland in 2019 (Szymańska-Czerwińska et al., 2019), and it has also been found in Brazil (Mioni *et al*., 2019). According to a study conducted by Polish scientists, MST61 shows the highest similarity to the MST20 genotype, as the MST61 genotype differs from MST20 by a single nucleotide deletion in the Cox37 intergenic region (Szymańska-Czerwińska et al., 2019). Bacteria with the MST20 genotype contain the QpH1 plasmid (Glazunova *et al*., 2005). Elsewhere in the world, there are no data on the virulence of the MST61 strain. The isolates with plasmid QpH1 and other plasmids have been evaluated as a cause for endocarditis, vascular infections, or abortion in the human population in the Netherlands (Angelakis *et al*., 2013). Very similar genotypes with only one allele difference have been found outside Europe (Brazil and United States of America) (Fig. 4). Previous studies have also shown that genotypes are not strictly related to geographical regions. Pathogens may have been exchanged between Poland and Latvia, for example as a result of international animal trade, but these genotypes may just as well have spread and stabilised in Europe in a natural way.

CONCLUSIONS

The prevalence of Q-fever in ruminants in the territory of Latvia was assessed as the percentage of positive sheds by the presence of *C. burnetii* DNA in milk samples, and aborted foetuses, and also by the presence of *C. burnetii* antibodies in serum samples from cows that suffered abortion in 2018–2020. In comparison with the previous study from 2012–2015, the infection levels remained approximately the same according to *C. burnetii* DNA presence in milk and presence of *C. burnetii* antibodies in serum samples from

cows that suffered abortion — 11.02% and 12.63% vs. 10.70% and 13.40%, respectively. The situation has not deteriorated, but has not improved either. We should note the absence of a comprehensive surveillance programme leading to specific control and biosecurity measures. Taking into account that the spatial distribution of Q-fever has increased in Latvia, and in the neighbouring countries the prevalence of the disease is significantly higher and ruminant import from abroad was indicated as a significant risk factor, it is advised to strengthen the Q-fever prevention measures at the country level in order to prevent disease spread among ruminant sheds and to avoid large human outbreaks in the future.

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COXIELLA BURNETII IZDALĪŠANĀS AR SLAUCAMO GOVJU PIENU UN Q-DRUDŽA SASTOPAMĪBA ATGREMOTĀJIEM LATVIJĀ AR UZSVARU UZ ABORTA GADĪJUMIEM

Q-drudzis ir plaši izplatīts visā pasaulē. Šī pētījuma mērķis bija novērtēt Q-drudža infekcijas izplatību Latvijā slaucamo govju ganāmpulkos 2018.–2020. gadā salīdzinājumā ar 2012.–2015. gadu. Tika novērtēta *Coxiella burnetii* DNS izdalīšanās ar pienu, kā arī tika veikta no aborta cietušo govju asins paraugu pārbaude, lai konstatētu antivielas pret Q-drudzi, un abortēto augļu pārbaude uz *C. burnetii* DNS klātbūtni. Pētījumā tika iekļauti arī klīniski veselu liellopu, kā arī aitu un kazu asins paraugi. Kopumā 18,34% piena paraugu bija pozitīvi, kas atbilst 11,02% no visām pārbaudītajām novietnēm. Kopumā 20,62% seruma paraugu no liellopiem, kuri cietuši no abortiem, bija pozitīvi vai aizdomīgi, kas veido 12,63% no visām novietnēm. Tikai 3,33% klīniski veselu liellopu un 3,42% aitu un kazu seruma paraugu bija pozitīvi. Vislielākais seroloģiski pozitīvo un aizdomīgo paraugu īpatsvars tika novērots slaucamajām govīm, kuras piedzīvojušas abortu, vecumā no trim līdz deviņiem gadiem, kā arī pirmajā un trešajā grūsnības trimestrī. Tika konstatēts viens dominējošs *C. burnetii* genotips (MST61). Atgremotāju imports no ārvalstīm, kā arī piena liellopu populācijas blīvums tika identificēti kā nozīmīgi riska faktori.